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Decreased number and bactericidal activity against *Staphylococcus aureus* of the resident cells in milk of dairy cows during early lactation

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Summary. Phagocytic and bactericidal activity of polymorphonuclear neutrophil leukocytes (PMN) isolated from blood and milk, against *Staphylococcus aureus*, was compared between groups of six healthy dairy cows in early, mid- and late lactation using a bacteriological assay. PMN were isolated from blood with a high degree of purity, but the cells isolated from milk contained variable amounts of macrophages (MΦ) and lymphocytes (L). The results were therefore calculated using the percentage PMN in order to evaluate phagocytosis and killing by PMN only. Blood PMN phagocytosed 82% *Staph. aureus* and milk PMN 43% on average and there was no significant difference between the different stages of lactation. The bactericidal activity of blood PMN against *Staph. aureus* was 36 ± 8% in early lactation (significantly different from mid lactation, $P < 0{\cdot}05$), 64 ± 10% in mid lactation and 53 ± 6% in late lactation. Milk PMN killed only 6 ± 3% *Staph. aureus* in early lactation (significantly different from mid lactation, $P < 0{\cdot}01$), 27 ± 3% in mid lactation and 20 ± 9% *Staph. aureus* in late lactation. The ratio of the bactericidal activity of milk to blood PMN was 0·08, 0·43 and 0·22 in early, mid- and late lactation, respectively. In addition to the decreased function, the number of cells in milk (somatic cell count, SCC) was also 60% lower in early lactation than in mid lactation cows ($P < 0{\cdot}01$). Our results suggest an impairment of blood and milk-resident PMN bactericidal activity against *Staph. aureus* and a decreased number of milk-resident PMN in dairy cows at the onset of lactation.

Keywords: Bovine mastitis, *Staphylococcus aureus*, polymorphonuclear neutrophil leukocyte, phagocytosis, intracellular killing

Inflammation of the mammary gland is characterised by increased somatic cell counts (SCC), resulting from the massive diapedesis of polymorphonuclear neutrophil leukocytes (PMN) into the udder. The increased incidence of severe coliform mastitis during early lactation has been correlated with a decreased number and function of blood PMN (Burvenich *et al.* 1994). Unlike blood PMN, it is unclear what role milk-resident PMN could play in intramammary immunity. The association between decreased milk SCC and risk for clinical mastitis, suggests that at least the number

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of milk PMN could play a role in defence against intramammary pathogens (Shuster *et al.* 1996; Peeler *et al.* 2000). The main purpose of this study was to compare the phagocytic and bactericidal activity of milk-resident PMN against *Staph. aureus*, in addition to SCC, between early, mid- and late lactating cows using blood PMN function as a reference.

In comparison with blood PMN, milk PMN could be considered as exhausted cells approaching death because their phagocytic and bactericidal activity is decreased (Russell *et al.* 1977; Paape *et al.* 1977), intracellular glycogen stores are depleted (Naidu & Newbould, 1973), their half-life time is very short (± 8 h), and they show apoptotic features (Van Oostveldt *et al.* 1999). Nevertheless, several studies suggest that milk-resident cells may exert a significant antibacterial activity. For example, phagocytosis of *Staph. aureus* by milk PMN was only 10% lower than blood PMN (Dulin *et al.* 1988). Bovine milk cells consist of macrophages (MΦ), PMN, lymphocytes (L) and a small number of epithelial cells and the qualitative composition of SCC is dependent on the stage of lactation (Concha, 1988). The relative role of each individual cell type in the defence against intramammary infections is poorly understood. An additional objective of this study was therefore to investigate the relative contribution of milk-resident PMN and MΦ to phagocytosis and killing of *Staph. aureus*. Our experiments were focused on the two types of phagocytes in milk: PMN and MΦ.

materials and methods

Experimental animals and sampling procedure

Eighteen healthy Holstein-Friesian cows from the Ghent University experimental dairy farm (Biocentrum Agri-Vet) were selected on the basis of 2 consecutive bacteriologically negative milk samples and SCC below 1×10^5/ml milk per individual quarter. Three different stages of lactation were investigated: early lactation cows (6–18 d after parturition, $n = 6$), mid-lactation cows (150–202 d after parturition, $n = 6$) and late-lactation cows (264–325 d after parturition, $n = 6$). For comparison of the phagocytic and bactericidal activity of milk PMN and MΦ in Percoll® (Pharmacia Biotech AB, Uppsala Sweden)-isolated fractions (see below), six healthy mid-lactation cows were selected. Blood samples were collected aseptically from the external jugular vein into evacuated tubes (Laboratoire EGA, Nogent-le Roi France) containing 125 I.U. heparin. Cisternal milk samples (1 l) were always collected aseptically from one quarter using a sterile teat cannulla after cleaning and disinfection of the teats (Vangroenweghe *et al.* 2000). Milk samples were taken immediately prior to the morning milking at 08.00. Milk was stored on ice until processed. No stimulation of the mammary gland was used prior to milk collection.

Isolation of neutrophil leukocytes from blood and milk

All materials and reagents used for the isolation of blood and milk PMN were sterile. Leukocytes were isolated from blood according to Carlson & Kaneko (1973). After counting the cells using an electronic particle counter (Coulter counter Z2, Coulter Electronics, Luton UK) and determination of the viability and percent PMN as described below, the suspension was adjusted to a concentration of 5×10^6 viable blood PMN/ml with Hank's Balanced Salt Solution (HBSS) containing Ca^{2+} and Mg^{2+} (GIBCO Life Technologies, Paisley Scotland) supplemented with 1 g Bovine Serum Albumin/l (BSA; Sigma Chemicals, St. Louis MO USA) and 25 mM-HEPES buffer (HBSS-BSA; Sigma).

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The milk samples were maintained on melting ice following sampling and during the isolation procedure. Initial volumes (2 l) of milk were processed using a high-capacity centrifuge (RC-3BP, Sorvall, Newtown CT USA), after 50 % dilution with PBS. Fat was carefully removed after the first centrifugation (1000 ***g***, 4 °C, 15 min) and the pellet was washed twice with PBS and centrifuged at 500 ***g*** for 15 min and 600 ***g*** for 10 min. After counting the isolated milk cells using an electronic particle counter (Coulter counter Z2) and determination of the viability as described below, the cells were finally resuspended to a concentration of 5×10^6 viable milk cells/ml with HBSS-BSA.

Macrophage and PMN-enriched fractions from milk were obtained by layering isolated milk cell suspensions on a discontinuous Percoll® (Pharmacia) gradient with densities of 1092 and 1071 g/l and centrifugation at 1000 ***g*** for 15 min. The bottom and upper fractions, predominantly consisting of PMN and MΦ, respectively, were collected separately, the purity of the suspensions was determined light microscopically at magnification ×1000 on May-Gruenwald's eosin-methylene blue solution (Merck Diagnostica, Darmstadt Germany) stained smears and the phagocytic and bactericidal activity against *Staph. aureus* was quantified as described below.

Viability and identification of leukocytes

The viability of PMN was determined by flow cytometry (FACScan, Becton Dickinson Immunocytometry Systems, San José CA USA) after addition of 10 μl propidium iodide (PI, 250 μg/ml PBS; Sigma) to 490 μl isolated cells (1×10^6 PMN/ml). Red fluorescence was registered at 650 nm with 460 V applied to the photomultiplier and the 488-nm excitation wavelength was used. The red fluorescence of PMN selected in the forward scatter – side scatter dot plot was evaluated with the CellQuest software (Becton Dickinson). The threshold value for live and dead cells was determined in a sample of isolated cells without PI. This method corresponded to the microscopical trypan blue exclusion method. Flow cytometry was more accurate than microscopy, with a CV of 5 % (10000 cells counted) compared to a CV of 28 % for the microscopical method (200 cells counted). Differential counts were performed on smears of isolated cells using light microscopy at magnification ×1000 after staining with May-Gruenwald's eosin-methylene blue solution. Plasma was added to the isolated cells in a 1 : 1 ratio in order to prevent lysis during preparation of the smears. Identification of the cells in milk was based on morphological characteristics as described by McDonald & Anderson (1981). Neutrophils were characterised by their multi-lobed or sometimes picnotic, dark blue stained nucleus. Macrophages typically had a large size, a vacuolated nucleus and contained lipids in their cytoplasm. Lymphocytes were either large with a low nucleus to cytoplasm ratio and a light blue stained nucleus or small with a high nucleus to cytoplasm ratio and a dark blue stained nucleus. Epithelial cells were identified as large, polygonal, uniformly stained light blue cells.

Analysis of milk composition: *somatic cell count*, *fat*, *protein and lactose*

SCC was determined with a fluoro-opto electronic method (Fossomatic 5000 cell counter, Foss Electric, Hillerød Denmark). Milk fat, protein and lactose content were determined by mid-infrared spectrophotometry (Fossomatic).

Table 1. *Cellular composition and phagocytic and bactericidal activity against* Staphylococcus aureus *Newbould 305 of 2 different fractions obtained after Percoll separation of cells isolated from milk. Purity is % PMN in the bottom fraction and % MΦ in the upper fraction*

(Values are means ± SEM for 6 healthy mid lactation cows)

	Fraction	
	Bottom (PMN)	Upper (MΦ)
Purity (%)	85 ± 3	78 ± 1
Phagocytosis (%)	96 ± 2	89 ± 6
Killing (%)	52 ± 6	31 ± 7

Phagocytic and bactericidal activity of blood and milk neutrophil leukocytes

Phagocytosis and killing of *Staph. aureus* Newbould 305 (Naidu & Newbould, 1975) was monitored by a bactericidal assay using sample cultivation according to Barta (1993) with modifications. Live bacteria (100 μl; 5×10^7/ml) were added to 500 μl viable PMN isolated from blood or viable cells isolated from milk (5×10^6/ml). The volume of the incubation mixture was adjusted to 1 ml with HBSS containing Ca^{2+} and Mg^{2+} supplemented with 1 g BSA/l and 25 mM-HEPES buffer. For the PMN isolated from blood and for the cells isolated from milk, respectively, 1 % zymosan-activated (and complement heat-inactivated) serum (ZAS) and 5 % complement heat-inactivated serum (V/V, final concentrations) were added. The bacteria to neutrophil ratio was 2 : 1. Incubation was carried out at 37 °C with end-over-end rotation for 60 min. The reaction was stopped by placing the tubes on ice. Ten microlites were sampled from the assay mixture immediately before and after 60 min and diluted with 5 ml distilled H_2O. Two further 10-fold dilutions were performed and plated out on Columbia sheep blood agar (Biokar Diagnostics, Beauvais France). Extracellular bacteria were separated by centrifugation (100 ***g***, 1 min), 10 μl was sampled and diluted as described above. The plates were incubated overnight at 37 °C after which colony counts were performed. Results from the bacteriological assay are expressed as the percentage of killed (% killing) and phagocytosed (% phagocytosis) *Staph. aureus* compared to the initially administrated amount of bacteria according to Barrio *et al.* (2000). A correction factor was included to account for bacterial growth during the assay. The results from the phagocytosis and bactericidal assay of PMN isolated from blood were calculated using % PMN with the following formula, where Y = % phagocytosis or % killing:

$$Y_{PMN} = \frac{Y_{\text{isolated cells}} \cdot 100}{\%\ \text{PMN}}$$

In the cells isolated from milk, the phagocyte population consisted of both PMN and MΦ. Macrophages had a similar phagocytic activity as PMN (Table 1). Thus, for phagocytosis, the results were calculated using % PMN and % MΦ with the following equation, where Y = % phagocytosis:

$$Y_{PMN} = \frac{Y_{\text{isolated cells}} \cdot \%\ \text{PMN}}{(\%\ \text{PMN} + \%\ \text{MΦ})}$$

The results from the bactericidal assay of milk PMN were calculated using the percentage PMN with the following formula, where Y = percentage killing. The 50 %

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lower bactericidal activity of MΦ than PMN was taken into account in this formula (Table 1):

$$Y_{PMN} = \frac{Y_{\text{isolated cells}} \cdot \%\ PMN}{[\%\ PMN + (\%\ M\Phi \times 0{\cdot}50)]}$$

The coefficient of variation of the bacteriological assay for phagocytosis and killing of *Staph. aureus* by blood PMN was 5% and 9%, respectively and for milk PMN 9% and 15%, respectively.

Contribution of milk neutrophils and macrophages to phagocytosis and killing of Staph. aureus

The phagocytic and bactericidal activity of pure PMN and MΦ was calculated using the lower (neutrophil-enriched) and upper (MΦ-enriched) fractions of isolated cells after Percoll separation. The following formulas were used, where Y = phagocytosis or bactericidal activity, Y_{PMN} = phagocytosis or bactericidal activity of pure PMN and $Y_{M\Phi}$ = phagocytosis or bactericidal activity of pure MΦ:

$$Y_{\text{upper}} = Y_{PMN} \cdot \%\ PMN + Y_{M\Phi} \cdot \%\ M\Phi$$

$$Y_{\text{bottom}} = Y_{PMN} \cdot \%\ PMN + Y_{M\Phi} \cdot \%\ M\Phi$$

Statistical analysis

Statistical analysis of the data was performed using a computer program (Statistix® 4.0, Analytical Software, Talahassee FL USA). Analysis of variance was performed using a mixed model with stage of lactation as a fixed factor and cows within stage of lactation as random factors:

$$Y = b_0 + b_1 X + e$$

where Y = blood or milk PMN function, b_0 = intercept, b_1 = coefficient for effect of the stage of lactation, X = stage of lactation and e = error term. The differences between early, mid- and late lactation were evaluated using a least significant differences comparison of means. Variances between cows within one stage of lactation group were equal according to a Bartlett's test of equal variances, so that analysis of variance was allowed.

RESULTS

Purity and viability of neutrophil leukocytes isolated from blood and milk

The % PMN in the cells isolated from blood averaged 80% and was not significantly different between the stages of lactation. The % PMN in isolated cells from milk was 36 ± 8% in early lactation cows, 64 ± 7% in mid lactation cows and 70 ± 5% in late-lactation cows. The viability of blood PMN was 98 ± 1% in early and mid-lactation cows and 97 ± 1% in-late lactation cows. At 36%, the viability of milk PMN in early lactation cows was significantly ($P < 0{\cdot}01$) lower than in mid- and late lactation, which were 64% and 70%, respectively.

Milk composition

SCC was lowest in early lactation cows, a value of $39 \pm 12 \times 10^3$/ml milk. In mid- and late-lactation cows, SCC was $97 \pm 12 \times 10^3$ and $76 \pm 11 \times 10^3$/ml milk, respectively. SCC was significantly lower in early lactation cows than in mid- and late-

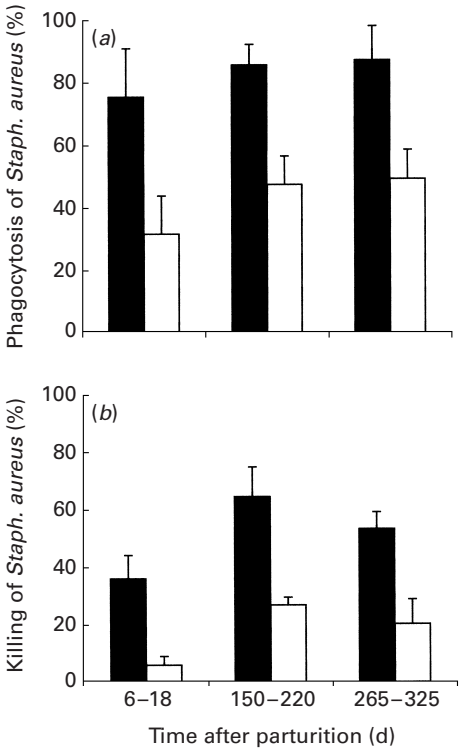

Fig. 1. (*a*) Phagocytosis and (*b*) Killing of *Staphylococcus aureus* Newbould 305 after incubation with neutrophils isolated from blood (solid bars) and milk (open bars) of healthy cows during different stages of lactation. Values are means ± SEM.

lactation cows ($P < 0{\cdot}01$). There were no significant differences in milk protein, fat and lactose content between the three stages of lactation. At $31 \pm 9\%$, the percentage isolated milk PMN was significantly lower in early lactation than in mid- ($66 \pm 3\%$) or late ($64 \pm 5\%$) lactation ($P < 0{\cdot}01$).

Contribution of milk neutrophils and macrophages to phagocytosis and killing of Staph. aureus

The percentage phagocytosis and killing by milk PMN and MΦ was calculated from Percoll-separated PMN and MΦ fractions of isolated cells (Table 1). Whereas the phagocytic activity of MΦ and PMN was similar, the bactericidal activity of MΦ was 50% lower than that of PMN. Phagocytic and bactericidal activity of MΦ was taken into account in order to calculate the phagocytic and bactericidal activity of milk PMN in isolated cell suspensions.

Phagocytosis of Staph. aureus *by blood and milk neutrophils*

Blood PMN phagocytosed $75 \pm 15\%$ *Staph. aureus* in early, $86 \pm 7\%$ in mid- and $84 \pm 9\%$ in late lactation (Fig. 1*a*). The percentage *Staph. aureus* phagocytosed by milk PMN was $32 \pm 12\%$, $47 \pm 9\%$ and $49 \pm 9\%$ for the early, mid- and late-lactation cows, respectively (Fig. 1*a*). The ratio of milk to blood PMN phagocytosis was 0·28 in early, 0·52 in mid- and 0·60 in late lactation. A significant positive correlation was found between the phagocytic activity of blood and milk PMN (Table 2).

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Table 2. *Correlations between blood and milk polymorphonuclear neutrophil leukocyte (PMN) phagocytosis and bactericidal activity against* Staphylococcus aureus *Newbould 305 in 18 healthy dairy cows*

PMN function	R	*P*
Phagocytosis of *Staph. aureus*	+0·81	$P < 0{\cdot}001$
Killing of *Staph. aureus*	+0·26	ns

ns, not significant; R, coefficient of correlation; *P*, significance level of the correlation.

Bactericidal activity of blood and milk neutrophils against Staph. aureus

The percentage killed *Staph. aureus* by blood PMN was $36 \pm 8\%$ for the early lactation cows, $64 \pm 10\%$ for the mid-lactation cows and $53 \pm 6\%$ for the late-lactation cows (Fig. 1*b*). The bactericidal activity of blood PMN was significantly lower in the early lactation cows compared to the mid-lactation cows ($P < 0{\cdot}05$). The percentage killed *Staph. aureus* by milk PMN was $6 \pm 3\%$ for the early lactation cows, $27 \pm 3\%$ for the mid-lactation cows and $20 \pm 9\%$ for the late-lactation cows. The bactericidal activity of milk PMN was significantly lower in the early lactation cows than in the mid-lactation cows ($P < 0{\cdot}05$) (Fig. 1*b*). The ratio of milk to blood PMN bactericidal activity was 0·08 in early, 0·43 in mid- and 0·22 in late lactation. There was no significant correlation between the bactericidal activity of blood and milk PMN (Table 2).

DISCUSSION

The present study was conducted to evaluate the phagocytic and bactericidal activity of blood and milk PMN from healthy cows during three stages of lactation. For that purpose, milk cells were collected from healthy mammary glands without prior stimulation. This approach contrasts with other studies in which the mammary gland has been challenged with lipopolysaccharide, oyster glycogen or an intramammary device in order to obtain a large number of cells, predominantly PMN, by induction of an inflammatory response (Paape *et al.* 1976; Saad, 1987; Dulin *et al.* 1988) or with studies investigating the immune function of milk cells in cows with mastitis (Piccinini *et al.* 1999). As a consequence of our experimental protocol, milk samples typically had a low SCC ($< 0^5$ cells/ml) and the isolated cells consisted of variable amounts of PMN, MΦ, L and epithelial cells. The original results from the bactericidal assay were therefore calculated using the PMN content of isolated milk cells in order to obtain the phagocytic and bactericidal activity of milk PMN. This approach allowed us to calculate the phagocytic and bactericidal activity of milk PMN from suspensions with a variable qualitative cellular composition. In this calculation, a correction factor was used for milk MΦ. Milk MΦ were as phagocytic as milk PMN but had a 50% lower bactericidal activity using complement-inactivated serum for opsonization. These findings are consistent with a previous study in which, under similar conditions of opsonization, phagocytosis by milk MΦ and PMN was equal and chemiluminescence (CL) activity – a parameter for oxygen-dependent bactericidal activity – was lower in milk MΦ than in PMN (Hallèn Sandgren *et al.* 1991). The normal phagocytic but low bactericidal activity of milk MΦ suggests that the primary effector cells for elimination of bacteria in the mammary gland are most likely PMN.

On average, 82% of the originally administered *Staph. aureus* were phagocytosed by blood PMN and 43% by milk PMN at a low bacteria to PMN ratio (2:1) in our

study. At a bacteria to PMN ratio of 20:1, 52% *Staph. aureus* were phagocytosed by blood PMN and 47% by milk PMN (Saad, 1987). Although a different opsonization could explain some of the difference between the results of our study and those of Saad (1987), it appears that PMN phagocytose less bacteria at a higher bacteria to PMN ratio. Indeed, when the ratio of bacteria to PMN was increased from 1:3 to 11:3, the number of phagocytosed bacteria was decreased (Williams *et al.* 1985). These *in vitro* experiments suggest that, at a given number of bacteria, the presence of a large amount of milk PMN may provide some protection of the mammary gland *in vivo* in comparison with low amounts of milk PMN. This hypothesis is supported by the findings of Shuster *et al.* (1996), who demonstrated that a moderately increased SCC before infection had a protective effect against induced *Escherichia coli* mastitis. In addition, recent evidence has been found to support the concept that critically reduced SCC are a risk factor for severe clinical mastitis in dairy herds (Peeler, 2000; Suriyasathaporn *et al.* 2000).

Complement-inactivated bovine serum was used to opsonize *Staph. aureus* Newbould 305 in this study. The presence of complement does not appear to be necessary for opsonization of *Staph. aureus* in order to be phagocytosed by bovine PMN (Rainard *et al.* 2000). Therefore, it can be accepted that sufficient opsonization condition were used in our study for phagocytosis of *Staph. aureus* Newbould 305 by bovine blood and milk PMN. The difference from the study of Ferrante *et al.* (1993), in which little killing of *Staph. aureus* was observed in the presence of complement-inactivated serum, might be explained by the use of a different *Staph. aureus* strain. For incubation of *Staph. aureus* Newbould 305 with blood PMN, 1% ZAS (which was also complement-inactivated) was used and for milk PMN, 5% complement-inactivated serum was used. The reason for these different opsonization conditions is that blood PMN are in a quiescent state whereas milk PMN are in an activated state. Therefore, ZAS – which contains C5a – was used with blood PMN. Neutrophils can be primed by this inflammatory mediator to enhance their functional activity (Rainard *et al.* 2000). The described opsonization conditions had been determined to be optimal for phagocytosis and killing of *Staph. aureus* by blood and milk PMN in preliminary experiments. Therefore, differences in phagocytic and bactericidal activity of blood and milk PMN between the stages of lactation were measured under optimal and constant opsonization conditions.

Phagocytic and bactericidal activity was significantly lower in milk than in blood PMN in all stages of lactation. A reduction of the phagocytic and bactericidal activity of milk PMN may result from diapedesis and ingestion of fat and casein (Paape & Wergin, 1977). That diapedesis of blood PMN through the blood-milk barrier causes a reduction of the phagocytic and oxidative burst activity was also demonstrated using an *in vitro* cell culture model (Smits *et al.* 1999). The negative correlation between intracellular survival of *Staph. aureus* and CL activity of bovine blood PMN (Williams *et al.* 1985), suggests that oxidative burst activity is important for the intracellular killing of *Staph. aureus*. The decreased bactericidal activity of milk PMN in comparison with blood PMN could thus be explained by a lower superoxide production measured by CL (Dulin *et al.* 1988) and a lower H_2O_2 production measured by flow cytometry (Salgar *et al.* 1991).

Phagocytosis of *Staph. aureus* by blood and milk PMN was not significantly decreased during early lactation in comparison with mid- and late lactation. Studies on blood PMN phagocytosis in dairy cows during early lactation have generated variable results: *Staph. aureus* phagocytosis was not changed (Berning *et al.* 1993) or decreased (Kehrli *et al.* 1989) and *E. coli* phagocytosis was not changed (Dosogne *et*

al. 1999). Ingestion of yeast cells by milk PMN was decreased during the second week after parturition (Guidry *et al*. 1976), whereas *Staph. aureus* phagocytosis by milk PMN was increased 5 d post partum (Berning *et al*. 1993). These differences can be explained by different opsonization, bacteria to PMN ratios, bacterial strains, and by the marked variation among cows in milk PMN phagocytosis (Paape *et al*. 1978). The opsonic activity of milk, which is increased in early lactation, may compensate for a decreased phagocytic capacity of PMN (Hill *et al*. 1983*b*). Moreover, during mastitis, opsonins in milk are increased (Jain & Lasmanis, 1978; Hill *et al*. 1983*a*). Therefore it has been concluded that opsonization of bacteria is not a critical factor in the susceptibility to intramammary infections with *E. coli* (Hill, 1981). It has also been concluded that PMN phagocytosis is not critically reduced during the early post-partum period with respect to the sensitivity to a severe clinical response during *E. coli* mastitis (Dosogne *et al*. 1997). This hypothesis is supported by results from the present study. Indeed, whereas phagocytosis is necessary, it is insufficient to eliminate bacteria without proper activation of the bactericidal system. In the case of *Staph. aureus* mastitis, the inefficient killing by milk PMN is a potential source of reinfection (Daley *et al*. 1991) whereas during *E. coli* mastitis, the decreased blood PMN oxidative burst activity contributes to the severity of the clinical symptoms (Heyneman *et al*. 1990). Therefore, intracellular killing following phagocytosis by PMN may be a more reliable parameter for estimation of the defence capacity against intramammary infections than phagocytosis alone.

The bactericidal activity of blood and milk PMN against *Staph. aureus* was significantly lower in early than in mid- and late lactation. This decreased bactericidal activity could be explained by the reduced oxidative burst activity, as observed in several other studies (Detilleux *et al*. 1995; Hoeben *et al*. 2000). However, the CL activity of blood PMN was only reduced by 8% in dairy cows during the first 2 weeks post partum in comparison with the dry period (Hoeben *et al*. 2000). In our study, the bactericidal activity of blood and milk PMN was reduced in early lactation by 44% and 78%, respectively, in comparison with mid lactation. Therefore, our findings suggest that not only oxygen-dependent killing but also other bactericidal mechanisms may be impaired during early lactation. Indeed, in human PMN, both oxygen-dependent and oxygen-independent mechanisms are involved in the intracellular killing of *Staph. aureus* (Edwards *et al*. 1987; Hermann *et al*. 1990; Hampton *et al*. 1996).

A significant positive correlation was found between milk and blood PMN phagocytosis and, although the correlation was not significant, it seems reasonable to suggest that there was also a relation between milk and blood PMN bactericidal activity. These results are supported by a previous study, in which the phagocytic capacity of blood PMN was reflected by the activity of milk PMN (Saad, 1987); in this study an intramammary device was used in order to obtain a large amount of PMN from milk. Together, these results suggest that the relationship between blood and milk PMN function in cows with unstimulated mammary glands is maintained during inflammation of the mammary gland. In addition, a lower ratio of milk to blood PMN bactericidal activity was found in early lactation in comparison with mid- and late lactation. This suggests that the inhibitory effects of diapedesis and ingestion of protein and fat on the bactericidal activity of blood PMN may be dependent on the stage of lactation.

In conclusion, we demonstrated a decreased bactericidal activity of both blood and milk PMN against *Staph. aureus* in cows during early lactation in addition to a decreased SCC and percentage PMN in milk. These findings suggest a significant

impairment of the intramammary aspecific defence mechanism related to milk cells in dairy cows during early lactation.

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REFERENCES

Barrio, B., Vangroenweghe, F., Dosogne, H. & Burvenich, C. 2000 Decreased neutrophil bactericidal activity during phagocytosis of a slime-producing *Staphylococcus aureus* strain. *Veterinary Research* **31** 603–609

Barta, O. 1993 Phagocytosis and killing of bacteria monitored by sample cultivation. In: Veterinary Clinical Immunology Laboratory. Bar-Lab. Inc., Blacksburg, USA, Chapter B3-IV

Berning, L. M., Paape, M. J. & Peters, R. R. 1993 Functional variation in endogenous and exogenous immunoglobulin binding to bovine neutrophils relative to parturition. *American Journal of Veterinary Research* **54** 1145–1153

Burvenich, C., Paape, M. J., Hill, A. W., Guidry, A. J., Miller, R. H., Heyneman, R., Kremer, W. D. J. & Brand A. 1994 Role of the neutrophil leukocyte in the local and systemic reactions during experimentally induced *E. coli* mastitis in cows immediately after calving. *Veterinary Quarterly* **16** 45–49

Carlson, G. P. & Kaneko, J. J. 1973 Isolation of leukocytes from bovine peripheral blood. *Proceedings of the Society for Experimental Biological Medicine* **142** 853–856

Concha, C. 1988 Cell types and their immunological functions in bovine mammary tissues and secretions – a review of the literature. *Nordisk Veterinar Medizin* **38** 257–272

Daley, M. J., Oldham, E. R., Williams, T. J. & Coyle, P. A. 1991 Quantitative and qualitative properties of host polymorphonuclear cells during experimentally induced *Staphylococcus aureus* mastitis in cows. *American Journal of Veterinary Research* **52** 474–479

Detilleux, J. C., Kehrli, M. E., Stabel, J. R., Freeman, A. E. & Kelley, D. H. 1995 Study of immunological dysfunction in periparturient Holstein cattle selected for high and average milk production. *Veterinary Immunology and Immunopathology* **44** 251–267

Dosogne, H., Burvenich, C., van Werven, T., Roets, E., Noordhuizen-Stassen, E. N. & Goddeeris, B. 1997 Increased surface expression of CD11b receptors on polymorphonuclear leukocytes is not sufficient to sustain phagocytosis during *Escherichia coli* mastitis in early postpartum dairy cows. *Veterinary Immunology and Immunopathology* **60** 47–59

Dosogne, H., Burvenich, C., Freeman, A. E., Kehrli, M. E., Detilleux, J. C., Sulon, J., Beckers, J.-F. & Hoeben, D. 1999 Pregnancy-associated glycoprotein and decreased polymorphonuclear leukocyte function in early post-partum dairy cows. *Veterinary Immunology and Immunopathology* **67** 47–54

Dulin, A. M., Paape, M. J. & Nickerson, S. C. 1988 Comparison of phagocytosis and chemiluminescence by blood and mammary gland neutrophils from multiparous and nulliparous cows. *American Journal of Veterinary Research* **49** 172–177

Edwards, S. W., Say, J. E. & Hart, C. A. 1987 Oxygen-dependent killing of *Staphylococcus aureus* by human neutrophils. *Journal of Genetic Microbiology* **133** 3591–3597

Ferrante, A., Martin, A. J. & Bates, E. J. 1993 Killing of *Staphylococcus aureus* by tumor necrosis factor-alpha-activated neutrophils. The role of serum opsonins, integrin receptors, respiratory burst, and degranulation. *Journal of Immunology* **151** 4821–4828

Guidry, A. J., Paape, M. J. & Pearson, R. E. 1976 Effects of parturition and lactation on blood and milk cell concentrations, corticosteroids, and neutrophil phagocytosis in the cow. *American Journal of Veterinary Research* **37** 1195–1200

Hallén Sandgren, C., Nordling, K. & Björk, I. 1991 Isolation and phagocytic properties of neutrophils and other phagocytes from nonmastitic bovine milk. *Journal of Dairy Science* **74** 2965–2975

Hampton, M. B., Kettle, A. J. & Winterbourn, C. C. 1996 Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of *Staphylococcus aureus* by neutrophils. *Infection and Immunity* **64** 3512–3517

Hermann, M., Jaconi, M. E., Dahlgren, C., Waldvogel, F. A., Stendahl, O. & Lew, D. P. 1990 Neutrophil bactericidal activity against *Staphylococcus aureus* adherent on biological surfaces. Surface-bound extracellular matrix proteins activate intracellular killing by oxygen-dependent and -independent mechanisms. *Journal of Clinical Investigations* **86** 942–951

Heyneman, R., Burvenich, C. & Vercauteren, R. 1990 Interaction between the respiratory burst activity of neutrophil leukocytes and experimentally induced *Escherichia coli* mastitis in cows. *Journal of Dairy Science* **73** 985–994

Hill, A. W. 1981 Factors influencing the outcome of *Escherichia coli* mastitis in the dairy cow. *Research in Veterinary Science* **31** 107–112

Hill, A. W., Heneghan, D. J., Field, T. R. & Williams, M. R. 1983*a* Increase in specific opsonic activity in bovine milk following experimental *Escherichia coli* mastitis. *Research in Veterinary Science* **35** 222–226

Hill, A. W., Heneghan, D. J. & Williams, M. R. 1983*b* The opsonic activity of bovine milk whey for the phagocytosis and killing by neutrophils of encapsulated and non-encapsulated *Escherichia coli*. *Veterinary Microbiology* **8** 293–300

Hoeben, D., Monfardini, E., Opsomer, G., Dosogne, H., de Kruif, A., Beckers, J.-F. & Burvenich, C. 2000 Chemiluminescence of bovine polymorphonuclear leukocytes during the periparturient period and relation with metabolic parameters and bovine pregnancy-associated glycoprotein. *Journal of Dairy Research* **67** 249–259

Jain, N. C. & Lasmanis, J. 1978 Phagocytosis of serum-resistant and serum-sensitive coliform bacteria (Klebsiella) by bovine neutrophils from blood and mastitic milk. *American Journal of Veterinary Research* **39** 425–427

Kehrli, M. E., Nonnecke, B. J. & Roth, J. A. 1989 Alterations in bovine peripheral blood neutrophil function during the periparturient period. *American Journal of Veterinary Research* **50** 207–214

McDonald, J. S. & Anderson, A. J. 1981 Total and differential somatic cell counts in secretions from noninfected bovine mammary glands: the peripartum period. *American Journal of Veterinary Research* **42** 1366–1368

Naidu, T. G. & Newbould, F. H. 1973 Glycogen in leukocytes from bovine blood and milk. *Canadian Journal of Comparative Medical Veterinary Science* **37** 47–55

Naidu, T. G. & Newbould, F. H. 1975 Significance of beta-hemolytic *Staph. aureus* as a pathogen to the bovine mammary gland. *Zentralblatt fur Veterinarmedizin* [B] **22** 308–317

Paape, M. J., Pearson, R. E. & Schultze, W. D. 1978 Variation among cows in the ability of milk to support phagocytosis and in the ability of polymorphonuclear leukocytes to phagocytose *Staphylococcus aureus*. *American Journal of Veterinary Research* **39** 1907–1910

Paape, M. J., Pearson, R. E., Wergin, W. P. & Guidry, A. J. 1976 Enhancement of chemotactic response of polymorphonuclear leukocytes into the mammary gland and isolation from milk. *Journal of Dairy Science* **60** 53–62

Paape, M. J. & Wergin, W. P. 1977 The leukocyte as a defence mechanism. *Journal of the American Veterinary Medicine Association* **170** 1214–1223

Peeler, E. J., Green, M. J., Fitzpatrick, J. L., Morgan, K. L. & Green, L. E. 2000 Somatic cell counts – there is a lower limit. Proceedings of the British Mastitis Conference, Shepton Mallet, Institute for Animal Health/Milk Development Council, p. 87

Piccinini, R., Bronzo, V., Moroni, P., Luzzago, P. & Zecconi, A. 1999 Study on the relationship between milk immune factors and *Staphylococcus aureus* intramammary infections in dairy cows. *Journal of Dairy Research* **66** 501–510

Rainard, P., Riollet, C., Poutrel, B. & Paape, M. J. 2000 Phagocytosis and killing of *Staphylococcus aureus* by bovine neutrophils after priming by tumor necrosis factor-α and the des-arginine derivative of C5a. *American Journal of Veterinary Research* **61** 951–959

Russell, M. W., Brooker, B. E. & Reiter, B. 1977 Electron microscopic observations of the interactions of casein micelles and milk fat globules with bovine polymorphonuclear leukocytes during phagocytosis of staphylococci in milk. *Journal of Comparative Pathology* **87** 43–51

Saad, A. M. 1987 Flow cytometric measurement of bovine milk neutrophil phagocytosis. *Acta Veterinaria Scandinavica* **28** 333–342

Salgar, S. K., Paape, M. J., Alston-Mills, B. & Miller, R. H. 1991 Flow cytometric study of oxidative burst activity in bovine neutrophils. *American Journal of Veterinary Research* **52** 1201–1207

Shuster, D. E., Lee, E. K. & Kehrli, M. E. 1996 Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving, compared with cows at mid lactation. *American Journal of Veterinary Research* **57** 1569–1575

Smits, E., Burvenich, C., Guidry, A. J., Heyneman, R. & Massart-Leën, A. 1999 Diapedesis across mammary epithelium reduces phagocytic and oxidative burst of bovine neutrophils. *Veterinary Immunology and Immunopathology* **68** 169–176

Suriyasathaporn, W., Schukken, Y. H., Nielen, M. & Brand, A. 2000 Low somatic cell count: a risk factor for subsequent clinical mastitis in a dairy herd. *Journal of Dairy Science* **83** 1248–1255

Vangroenweghe, F., Mehrzad, J., Bossuyt, R., Dosogne, H. & Burvenich, C. 2000 Influence of milk sampling procedures on cell functions of milk cells. IDF Symposium on Immunology of Ruminant Mammary Gland. Stresa, Italy. pp. 48–54

Van Oostveldt, K., Burvenich, C., Paape, M. J. & Meyer, E. 1999 The effect of diapedesis on the apoptotic response of isolated bovine neutrophils. Cell Biology International. Spring Meeting Apoptosis. Ghent, Belgium (abstr.)

Williams, M. R., Craven, N., Field, T. R. & Bunch, K. J. 1985 The relationship between phagocytosis and intracellular killing of *Staphylococcus aureus* by bovine neutrophils. *British Veterinary Journal* **141** 362–371